

GRB14 AND THE INSULIN RECEPTOR AND ALSO SCREENING FOR
NOVEL MEDICINAL PRODUCTS

5 The present invention relates to the use of the Grb14 protein and of homologous adapter proteins (proteins of the Grb7 family), as a tool for screening for molecules intended for treating diseases involving insulin.

10 Insulin, which is the principal hormone for the regulation of energy metabolism, is the only blood-glucose-lowering hormone in the body; it stimulates the transport of glucose and its use by peripheral tissues (skeletal muscles and adipose tissue) and inhibits the endogenous production of glucose by the liver.

15 Insulin acts through a receptor which is expressed at the plasma membrane of cells. This receptor is part of the family of receptors with tyrosine kinase activity, which are characterized by the presence of an
20 intracellular domain which bears the catalytic activity. Binding of the ligand induces dimerization of the receptors, activation of the tyrosine kinase domain and phosphorylation (autophosphorylation and transphosphorylation) of specific tyrosine residues present in
25 the cytosolic component of the receptors (Ullrich. A. et al. (1990) *Cell*, 61, 203-212).

30 The insulin receptor has the particularity of being present in a naturally dimerized form. The binding of the insulin to the extracellular α subunit induces conformation modifications which result in the activation of the kinase domain borne by the β subunit of the receptor, and in its autophosphorylation, which is required for complete activation of the receptor.
35 The insulin receptor activated in this way phosphorylates intracellular proteins which are used as insulin signal effectors.

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Specifically, the transduction of a signal inside the cell, after a receptor with tyrosine kinase activity has been stimulated, makes use of protein-protein interaction cascades which result in a metabolic or mitogenic effect, and in which the molecular adapters have a preferred role. Through their protein interaction domains, the adapters enable the recruitment of successive effectors, constituting the signalling pathways.

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Among the various relays between the insulin receptor and its intracellular effectors, the most well characterized adapter proteins are IRS-1, IRS-2 (*Insulin Receptor substrate-1 and 2*) and Shc (*Src and collagen homologous protein*) (White M.F. et al. (1994) *J. Biol. Chem.*, **269**, 1-4; Waters S.B., et al. (1996) *Trends Cell Biol.*, **6**, 1-4). They are not specific for insulin-sensitive tissues and are also phosphorylated both after the activation of other tyrosine kinase receptors and after that of cytokine receptors or G protein-coupled receptors (Bonfini L. et al. (1996) *Trends Biochem. Sci.*, **21**, 257-261; Souza S.C. et al. (1994) *J. Biol. Chem.*, **269**, 30085-30088; Argetsinger L.S. et al. (1995) *J. Biol. Chem.*, **270**, 14685-14692; Plataniias L.C. et al. (1996) *J. Biol. Chem.*, **271**, 278-282; Velloso L.A. et al. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 12490-12495; Kowalski-Chauvel A. et al. (1996) *J. Biol. Chem.*, **271**, 26536-26361).

Thus for example, the Shc protein binds to the activated insulin receptor, is then phosphorylated and recruits the Grb2 adapter which binds to phosphotyrosine residues of Shc, through its SH2 domain, and binds, via an SH3 domain, to the nucleotide exchanger Sos, which will itself enable the activation of Ras (Schlessinger, J. (1993), *Trends Bioch. Sci.*, **18**, 273-275).

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Recently, novel adapter proteins which may be specifically involved in insulin signal transduction have been cloned by interaction with the insulin receptor using the double-hybrid system, in particular
5 various isoforms of the Grb10 protein from humans and from mice (Liu F. et al. (1995) *Proc. Natl. Acad. Sci. USA*, 92, 10287-10291; O'Neill T.J. et al. (1996) *J. Biol. Chem.*, 271, 22506-22513; Frantz J.D. et al. (1997) *J. Biol. Chem.*, 272, 2659-2667).

10 Even more recently, the inventors have cloned the rat rGrb14 and rGrb7 proteins by interaction with the insulin receptor using the double-hybrid system (Kasus-jacobi et al. (1998) *J. Biol. Chem.*, 273, 26026-26035).

15 The mGrb10, hGrb10, hGrb14 and rGrb14 proteins belong to the same family of adapter proteins, the first known member of which is the Grb7 protein which binds to the receptor for EGF, for Ret and for PDGF (Margolis B.
20 (1992) *Proc. Natl. Acad. Sci. USA*, 89, 8894-8898). Hereinafter, the proteins of this family are termed proteins of the Grb7 family.

25 These proteins which have been cloned by interaction with activated insulin receptors appear to play an important role in insulin signal transduction.

Thus, the inventors have shown that the expression of the rGrb14 protein is very well correlated with the
30 sensitivity of tissues to insulin and that its overexpression in CHO-IR cells (Chinese Hamster Ovary cells expressing high levels of insulin receptors of human origin) inhibits the effects of insulin by decreasing the activation of IRS-1 without modifying
35 the autophosphorylation of the insulin receptor (Kasus-Jacobi et al. (1998) already cited).

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The adapter proteins of the Grb7 family are characterized by the succession of three domains:

- a proline-rich sequence named PP, close to the amino-terminal end,
- a central domain named PH (*Pleckstrin homology*) and
- a domain named SH2 (*Src homology 2*) at the carboxy-terminal end, known to interact with sequences containing phosphotyrosines (Ooi J. et al. (1995) *Oncogene*, **10**, 1621-1630; Margolis B. (1992) already cited; Daly R.J. (1996) already cited).

Besides these domains which have already been studied in other proteins, the inventors have revealed a novel domain on the rGrb14 protein, named PIR (*Phosphorylated Insulin Receptor Interacting Region*) corresponding to residues 340 to 437 of the protein; by comparison between the Grb7, Grb10 and Grb14 proteins, the inventors have shown that a 43 amino acid sequence corresponding to amino acids 365 to 470 of the rGrb14 protein is highly conserved throughout the family of these proteins (Kasus-Jacobi et al. (1998) already cited) and should play a specific role in the attachment of these proteins to the insulin receptor.

The PIR domain is homologous to the BPS domain (*Between PH and SH2*) (Kasus-Jacobi et al. (1998) already cited), recently demonstrated on the hGrb10 protein (He W. et al. (1998) *J. Biol. Chem.*, **273**, 6860-6867), and corresponds to amino acids 358-434 of the Grb14 protein.

The association between the activated insulin receptor and the proteins of the Grb7 family involves the two domains PIR and SH2. Depending on the Grb protein under consideration, the respective role of the two domains is more or less important. Specifically, it is

essentially PIR which is responsible for the binding of Grb14 to the insulin receptor (Kasus-Jacobi et al. (1998) already cited), whereas PIR and SH2 are involved in the interaction between Grb10 and the receptor (He et al (1998) already cited).

Several teams have shown that there are defects in phosphorylation of the insulin receptor and also modifications of the effects of insulin on the transport of glucose and on the activation of certain enzymes in obese or diabetic patients (Arner, P. et al., J. N. (1987), *Diabetologia*, **30**, 437-440; Caro, J. F. et al. (1987), *J. Clin. Invest.*, **79**, 1330-1337; Mandarino, L.J. (1989), *Diab. Metab. Rev.*, **5**, 475-486).

Mutations of the insulin receptor gene may lead, via various mechanisms, to a decrease in the tyrosine kinase activity of the receptor, thus contributing to the development of a condition of insulin resistance and to the institution of pathological conditions such as obesity and non-insulin-dependent diabetes (DNID) (Taylor, S.I. (1992), *Diabetes*, **41**, 1473-1490).

In conditions of insulin resistance, hyperglycemia develops when the endogenous secretion of insulin is no longer sufficient, and it is necessary to resort to insulin therapy in order to maintain carbohydrate homeostasis. After the diabetes has evolved for 10 years, severe complications are observed in 30% of cases. These complications, which are secondary to poor control of glycemia, have various very serious clinical implications (renal failure, necrosis and amputation of the lower limbs, blindness) which lead to a shortening of the life expectancy of the patients.

Normalization of the tyrosine kinase activity, when it is disturbed, may be envisioned either directly, using molecules which act on this enzyme (Levitzki et al.

(1995), *Science* **267**, 1782-1788) or indirectly, by inhibiting the interactions between the adapter proteins and the tyrosine kinase (Pendergast et al. (1993), *Cell*, **75**, 175-185).

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Now, the inventors have shown, surprisingly, that the binding, to the activated insulin receptor, of the PIR domain of the proteins of the family of Grb7 proteins (Grb14, Grb10 and Grb7), alone or associated with the SH2 fragment (PIR-SH2), inhibits the tyrosine kinase activity of said receptor.

The subject of the present invention is the use of a fragment consisting of the PIR domain or the PIR-SH2 domain of a protein of the family of Grb7 proteins, as a tool for screening for molecules intended for treating diseases involving insulin.

According to an advantageous embodiment of said use, said fragment is selected from the group consisting of the sequences: SEQ ID NO: 1-28 which correspond, respectively, to PIR fragments (residues 365-407 and residues 353-436) and to PIR-SH2 fragments (residues 365-538 and residues 353-538) of the rGrb14, hGrb14, mGrb10, hGrb10, rGrb7, hGrb7 and mGrb7 proteins.

For the purposes of the present invention, the numbering of the residues of the protein fragments is given with reference to the sequence of the rGrb14 protein after alignment.

Interestingly, the inventors have shown that the inhibitory effect of the Grb14 protein is reproduced by the purified GST-PIR and GST-PIR+SH2 fusion proteins, which are obtained by fusion of GST with the PIR domain or the PIR+SH2 domain of rGrb14. On the other hand, this inhibitory effect is not observed with the GST-SH2

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fusion protein obtained by GST fusion with the SH2 domain of rGrb14.

Unexpectedly, the inventors have shown that the PIR domain alone has an activity equivalent to that of the whole protein, whereas the PIR+SH2 domain has a much greater inhibitory effect than PIR expressed alone. Specifically, total inhibition of the tyrosine kinase activity of the insulin receptors is obtained when 0.3 µg of GST-PIR protein is added, whereas only 0.03 µg of GST-PIR+SH2 is necessary. It appears, therefore, that, while the SH2 domain has no inhibitory activity per se, on the other hand it greatly potentiates the effect of PIR.

In comparable fashion, the inventors have shown that the PIR and PIR+SH2 domains of Grb10 have an inhibitory effect on the tyrosine kinase activity of the insulin receptor. The SH2 domain of Grb10 does not, in itself, have an inhibitory effect, but it too potentiates the inhibition induced by PIR.

In addition, the inventors have shown that the insulin receptor is more sensitive to the inhibitory effect of Grb14 than to that of Grb10 and of Grb7, and that the effect may be obtained both with the whole protein and with the PIR domain or the PIR-SH2 domain.

The PIR and PIR-SH2 domains of the Grb14, Grb10 and Grb7 proteins therefore behave like endogenous inhibitors of the tyrosine kinase activity of the insulin receptor, which is an entirely novel function for molecular adapters. In fact, unlike the adapter proteins IRS-1, IRS-2 or Shc which are intermediates between the insulin receptor and cellular effectors, said domains of the proteins of the Grb7 family act directly on the tyrosine kinase activity of the insulin receptor.

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Consequently, the PIR and PIR-SH2 domains of the Grb14 protein and of the homologous adapter proteins (proteins of the Grb7 family) constitute potential
5 targets for medicinal products.

Specifically, compounds which are capable of increasing or of suppressing the interactions of the domains of said proteins may prevent or cure disorders of the
10 organism which are related to a modification of the activity of the kinase protein of the insulin receptor.

Consequently, the present invention therefore also relates to a method for detecting molecules capable of
15 stimulating or inhibiting (modulating) the tyrosine kinase activity of the insulin receptor, characterized in that it comprises:

- 20 a) bringing the activated insulin receptor into contact with a fragment consisting of the PIR domain or the PIR-SH2 domain of a protein of the family of Grb7 proteins, and the molecule to be tested, under conditions which allow binding of said fragment to said receptor,
- 25 b) adding a tyrosine kinase substrate,
- c) measuring the tyrosine kinase activity, and
- 30 d) determining the modulation (inhibition or stimulation) of the tyrosine kinase activity by comparison with a control consisting of the activated insulin receptor and said fragment.

35 In accordance with the invention, said fragment is preferably selected from the group consisting of the sequences SEQ ID NO: 1 to 28.

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According to an advantageous embodiment of said method,
prior to step a) above, a preselection of the molecules
capable of stimulating or inhibiting (modulating) the
interactions of a fragment consisting of the PIR domain
5 or the PIR-SH2 domain of a protein of the family of
Grb7 proteins, with the insulin receptor, is carried
out by:

- 1) immobilizing said fragment on a solid support,
- 10 2) bringing the molecule to be tested into contact
with said fragment, then
- 3) incubating with the labeled and pre-activated
15 insulin receptor, under conditions which allow binding
of said receptor to said fragment,
- 4) separating said labeled receptor not retained on
the support,
- 20 5) detecting the complex possibly formed between said
fragment and the activated insulin receptor, and
- 6) determining the effect of the molecule (inhibition
25 or stimulation of the fragment-receptor interaction),
by comparison with a control comprising said fragment
and the insulin receptor.

In order to allow said fragment to be immobilized on a
30 solid support, said fragment may, for example, be
expressed as a fusion with a protein such as GST.

Said receptor may, for example, be labeled with a
radioactive molecule or fused to a fluorescent protein
35 such as GFP (*Green Fluorescent Protein*).

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When said receptor is labeled with a fluorescent or radioactive molecule, the interaction between said fragment and said receptor is detected by reading the fluorescence or the radioactivity retained on the solid support.

A subject of the present invention is also the use of a molecule capable of binding to a fragment consisting of the PIR domain or the PIR-SH2 domain of a protein of the family of Grb7 proteins, and of inhibiting the tyrosine kinase activity of the insulin receptor, for manufacturing a medicinal product which can be used in the treatment of diseases involving insulin, in particular diabetes and obesity.

According to an advantageous embodiment of said use, said molecule is obtained using the method in accordance with the invention.

The compounds selected in this way can potentially be used for preventing or treating diseases involving insulin, such as for example diabetes and obesity or other pathological conditions characterized by insulin resistance, such as polycystic ovary syndrome (Legro, R.S. et al. (1998), *Rec. Progr. Hormone Res.*, **53**, 217-255) or syndrome X (Komers, R. et al., (1998), *Physiol. Res.*, **47**, 215-225).

Besides the arrangements above, the invention also comprises other arrangements which will emerge from the following description, which refers to examples of implementation of the method which is the subject of the present invention and also to the attached diagrams, in which:

- Figure 1 illustrates the alignment of the proteins of the family of Grb proteins. The percentages of amino acid identity of the domains are expressed relative to

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the homologous domain of rGrb14. PP: motif rich in proline residues, binding site for proteins containing SH3 domains; PH: pleckstrin homology domain, association with phospholipids or proteins; PIR
5 phosphorylated insulin receptor interacting region; SH2: domain allowing interaction with phosphotyrosine residues.

10 - Figure 2 illustrates the alignment of the sequences of the PIR domains of the proteins of the family of Grb proteins: rGrb14, hGrb14, hGrb10 and hGrb7. The numbering of the amino acids is given with reference to the sequence of the rGrb14 protein. The conserved amino acids are indicated with an asterisk. The conserved
15 domain corresponding to residues 365-407 of the Grb proteins is in gray.

20 - Figure 3 illustrates the sequence of the PIR-SH2 domains of the proteins of the family of Grb proteins: rGrb14, hGrb10 and hGrb7. The complete sequence of the PIR-SH2 domain (residues 353-538) of rGrb14 (SEQ ID NO: 4), hGrb10 (SEQ ID NO: 16) and hGrb7 (SEQ ID NO: 24) is given. The sequence of fragment 405-538 of the PIR-SH2 domain of rGrb14 (SEQ ID NO: 3), hGrb10 (SEQ ID NO: 15)
25 and hGrb7 (SEQ ID NO: 23) is underlined.

30 - Figure 4 illustrates the effect of the Grb proteins on the tyrosine kinase activity of insulin receptors; (●) GST-rGrb14; (▲) GST-mGrb10; (■) GST-rGrb7. The results are expressed as the percentage of the value obtained in the absence of added protein. Number of experiments = 4. The effects of the GST-mGrb10 and GST-rGrb7 proteins compared with those of the GST-rGrb14 protein show statistically significant differences,
35 indicated by * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

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- Figure 5 illustrates the inhibition of the tyrosine kinase activity of insulin receptors by the rGrb14 protein: (●) GST-rGrb14; (▲) GST-PIR of rGrb14; (☒) GST-SH2 of rGrb14; (■) GST-PIR+SH2 of rGrb14; (◇) GST-PIR+SH2 R464K of rGrb14. The results are expressed as the percentage of the value obtained in the absence of added protein. Number of experiments = 5. The effects of the various rGrb14 constructs compared with those of the whole GST-rGrb14 protein show statistically significant differences, indicated by * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

- Figure 6 illustrates the inhibition of the tyrosine kinase activity of insulin receptors by the various domains of mGrb10: (●) GST-mGrb10; (▲) GST-PIR of mGrb10; (☒) GST-SH2 of mGrb10; (■) GST-PIR+SH2 of mGrb10; (◇) GST-PIR+SH2 R547K of mGrb10. The results are expressed as a percentage of the value obtained in the absence of added protein. Number of experiments = 4. The effects of the various mGrb10 constructs compared with those of the whole GST-mGrb10 protein show statistically significant differences, indicated by: * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

- Figure 7 illustrates the inhibition of the tyrosine kinase activity of insulin receptors by the various domains of rGrb7: (●) GST-rGb7; (▲) GST-PIR of rGb7; (☒) GST-SH2 of rGrb7; (■) GST-PIR+SH2 of rGrb7. The results are expressed as a percentage of the value obtained in the absence of added protein. Number of experiments = 2. The effects of the various rGrb7 constructs compared with those of the whole GST-rGrb7 protein show statistically significant differences, indicated by: * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Example 1: Comparison of the effect of the rGrb14, mGrb10 and rGrb7 proteins on the tyrosine kinase activity of insulin receptors

5 1. Procedure:

Insulin receptors are partially purified from CHO-IR cells by passing a cell lysate over a weak germ lectin column and eluting the glycoproteins retained with
10 0.3 M N-acetylglucosamine. The insulin receptors thus purified are incubated in the presence of insulin (0 or 10^{-7} M) for 1 hour at room temperature. A buffer containing 20 μ M ATP, $MnCl_2$ and $MgCl_2$ ions and [γ - ^{32}P] ATP is then added so as to allow the receptors to
15 autophosphorylate, as are increasing amounts of the purified Grb proteins expressed as a fusion with GST. 30 minutes later, 15 μ g of a synthetic substrate, poly Glu-Tyr (4:1), are added. The tyrosine kinase activity of the receptors is measured by the incorporation of
20 radioactivity into the poly Glu-Tyr during 30 min.

2. Results:

They are represented in Figure 4.

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The addition of the GST-rGrb14 and GST-mGrb10 fusion proteins induces dose-dependent inhibition of the tyrosine kinase activity of the insulin receptors, and the highest concentrations allow total inhibition of
30 the enzyme. By comparison, the GST-rGrb7 protein enables a maximum of only 40% inhibition. The dose-response curve of the effect of GST-mGrb10 is shifted to the right compared to the curve of the effect of GST-rGrb14. 50% inhibition of the tyrosine kinase
35 activity of the receptors is obtained when using, respectively, 0.04 μ g of GST-rGrb14 and 0.13 μ g of GST-mGrb10. The tyrosine kinase activity of the insulin

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receptors is therefore more sensitive to the inhibitory effect of rGrb14 than to that of mGrb10.

These results show that the Grb proteins have inhibitory activity on the tyrosine kinase activity of insulin receptors and that the rGrb14 protein has the greatest inhibitory effect.

Example 2: Inhibitory effect of the various domains of rGrb14 on the tyrosine kinase activity of insulin receptors

1. Procedure:

The insulin receptors are partially purified as described in Example 1. The various domains of rGrb14 (rGrb14, PIR, SH2, PIR+SH2, PIR+SH2 R464K) are produced as a fusion with GST and purified. The inhibitory effect of these proteins on the tyrosine kinase activity of insulin receptors is analyzed as described in Example 1.

2. Results:

They are as represented in Figure 5.

The PIR domain exerts an inhibitory effect comparable to that of the whole rGrb14 protein, whereas the SH2 domain has no effect (in the same way as the protein deleted of the PIR+SH2 regions, results not shown). However, the PIR+SH2 domain has a much greater inhibitory effect than PIR alone or the whole protein (the maximum inhibitory effect is obtained by adding 0.03 µg of protein). This potentiation is suppressed by mutating the arginine 464 residue of the conserved FLVRES motif, which inactivates the SH2 domain, since the PIR+SH2 R464K domain has the same effect as PIR alone.

These results show that the inhibitory effect of rGrb14 on the tyrosine kinase activity of insulin receptors is due to the presence of PIR. The SH2 domain alone has no effect, but it potentiates the PIR effect.

Example 3: Inhibitory effect of the various domains of mGrb10 on the tyrosine kinase activity of insulin receptors

1. Procedure:

The procedure is identical to that described in Example 2.

2. Results:

They are represented in Figure 6.

The PIR domain exerts an inhibitory effect comparable to that exerted by the whole protein. The SH2 domain alone has no inhibitory action, but it potentiates the inhibition exerted by PIR (the dose-response curve of PIR+SH2 is shifted to the left). Mutating the arginine residue of the FLLRDS motif of the SH2 domain inhibits the potentiating effect of the PIR+SH2 domain (PIR+SH2 R547K mutant).

These results show that the PIR or PIR-SH2 domains of rGrb14 have a greater inhibitory effect than the PIR or PIR-SH2 domains of mGrb10.

Example 4: Inhibitory effect of the various domains of rGrb7 on the tyrosine kinase activity of the insulin receptors

1. Procedure:

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The procedure is identical to that described in Example 2.

2. Results:

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They are represented in Figure 7.

10 The PIR domain exerts an inhibitory effect comparable to that exerted by the whole protein. The SH2 domain alone has no inhibitory action, but it potentiates the inhibition exerted by PIR (the dose-response curve of PIR+SH2 is shifted to the left). The PIR and PIR-SH2 domains of rGrb7 have a lesser inhibitory effect compared to that of the PIR and PIR-SH2 domains of the
15 rGrb14 and mGrb10 proteins.

20 As emerges from the above, the invention is in no way limited to its methods of implementation, preparation and application which have just been described more explicitly; on the contrary, it encompasses all of the variants thereof which may occur to a person skilled in the art, without departing from the context or scope of the present invention.

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